# Targeted and Non-Targeted Analysis of *Ginkgo biloba* Extract (GbE) from 17 Suppliers

Collins, B.J.[1]; Rider C.[1]; Kerns, S.P.[2]; Sitzmann, B.D.[2]; Achey, C.M.[2]; Aillon, K.L.[2]; Waidyanatha, S.[1]

[1]Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

[2]MRIGlobal, Kansas City, MO 64110

## Abstract

Dietary supplements are often natural products consisting of complex mixtures of variable composition, making it difficult to extend the toxicological evaluation results for a single reference sample to similar products in the market. The NTP has begun to assess the chemical and biological parameters needed to establish sufficient similarity between natural products and GbE was selected as the first test article for this program. We evaluated the hydrolyzed and unhydrolyzed chemical similarity of 31 GbE samples procured from 17 suppliers using non-targeted HPLC-UV/ELSD. 25 GbE chromatograms had 15 to > 20 peaks in 4 categories: terpene lactones (TL), flavonol glycosides, flavonol aglycones (FA), and ginkgolic acids (GA); 6 samples yielded ≤ 1 peak. Targeted analysis for 12 constituents was performed on all 31 GbEs. Targets included FA (quercetin, kaempferol, isorhamnetin), rutin, and TL (bilobalide, ginkgolides A, B, C, and J) analyzed by HPLC-UV/ELSD, ginkgotoxin (GT) by HPLC-Fluorescence, and GA I and II by HPLC-MS/MS. Total TL was ≤ 20.04% (w/w). In hydrolyzed samples, total FA was ≤ 11.84%. FA were found in some unhydrolyzed samples indicating potential adulteration. Individual TL and FA varied widely between samples. GA (≤ 0.14% I; ≤ 0.22% II) was found in 23/31 samples. GT (≤ 0.01%) was found in 29/31 samples.

Differences between samples were related to the amounts of each constituent present and the presence or absence of aglycones indicating potential adulteration.

## Background

*Ginkgo biloba* extract (GbE) is a constituent of many commercially available herbal dietary supplements and is sold by a large number of vendors worldwide. GbE is an ethanolic extract of Ginkgo biloba leaves with a complex composition. Approximately 30-35% of the constituents of GbE are known. Standardized extracts contain 24% flavonol glycosides, which upon hydrolysis consist primary of quercetin, kaempferol, and isorhamnetin; and 6% terpene lactones, which are primarily ginkgolides A, B, C, and J, and bilobalide. In some GbE products pure flavonol aglycones are added to a substandard extract to meet the 24/6 requirement, while others are mixtures of source materials adjusted to give the appropriate 24/6 results. Commercially available GbE products in the US have levels of flavonol glycosides ranging from ~240 – 360 mg/g, terpene lactones from ~40 – 110 mg/g and ginkgolic acids from < 500 to ~90,000 µg/g[1]. The variable composition of GbE makes it difficult to extend the results of research on a single reference sample to similar products in the market. The NTP has begun to assess the chemical and biological parameters needed to establish sufficient similarity for GbE dietary supplements as compared to an authentic reference standard, and GbE was selected as the first case study for this program[6].

## Objectives

- Evaluate the use of non-targeted analysis to screen GbE samples from multiple suppliers. Compare non-targeted results to results for a NIST standard reference material (SRM).
- Use targeted analysis of 12 marker compounds to confirm the qualitative results from the non-targeted analysis. Compare targeted results to results for a NIST SRM.
- Authenticate 17 samples from 16 suppliers using HP-TLC (Alkemist). Compare targeted results to results for a NIST SRM.
- Compare NTP Tested lot to NIST SRM and commercially available GbE.

## Methods

### Non-Targeted LC/UV/ELSD* Analysis[2]

- Samples were analyzed on a Shimadzu LC-2010C HT liquid chromatograph coupled to an ELSD (Altech 3300)
- Column: Performance RP-18-e (100 x 4.6 mm)
- Mobile phases: Isopropyl alcohol (A), tetrahydrofuran (B), and 0.1% formic acid in water (C) were used with a flow rate of 1 mL/min and the following gradient: 5%A, 0%B, 95%C to 0%A, 13%B, 87%C in 15 minutes, then to 0%A, 40%B, 60%C in 35 minutes, then to 0%A, 75%B, 25% C in 20 minutes, hold for 5 minutes, then to 5%A, 0%B, 95%C in one minute, hold for 9 minutes. Total run time was 85 minutes.

### Targeted LC/UV/ELSD* Analysis[3]

- Samples were analyzed on a Shimadzu LC-2010C HT liquid chromatograph, coupled with an Altech 3300 ELSD (terpene lactones) or Shimadzu UV @267 nm (flavonol aglycones).
- Column: Phenomenex Prodigy 5µ ODS (3) 250 x 4.6 mm ID
- Mobile phase: water:methanol, 90:10 (v:v) with 0.25% formic acid (A), and methanol with 0.25% formic acid (B) with a flow rate of 1.0 mL/min and the following gradient: 85%A, 15%B, then to 62%A, 38%B in 23 minutes, then to 54%A, 46%B, in 2 minutes, hold for 30 minutes, then to 10%A, 90%B in 5 minutes, hold for 10 minutes, then to 85%A, 15%B in 2 minutes, hold 8 minutes. Total run time was 80 minutes.

### Targeted LC/MS/MS Analysis[4]

- Samples were analyzed on a Shimadzu LC-20AD liquid chromatograph coupled with a ABSciex Tandem Triple Quadrupole Mass Spectrometer run in negative turbo ionspray (–TIS) mode. Transitions monitored were 345 > 301 for ginkgolic acid I (15:1) and 373 > 329 for ginkgolic acid II (17:1).
- Column: Phenomenex Prodigy 5µ ODS (3) 250 x 4.6 mm ID.
- Mobile Phase: water:methanol, 900:100 (v:v) with 0.1% formic acid (A), and methanol with 0.1% formic acid (B) with a flow rate of 1.0 mL/min and the following gradient: 85%A, 15%B, hold 5 minutes, then to 5%A, 95%B in 10 minutes, hold 20 minutes, then to 85%A, 15%B, in 0.1 minute, hold for 4.9 minutes. Total run time was 40 minutes.

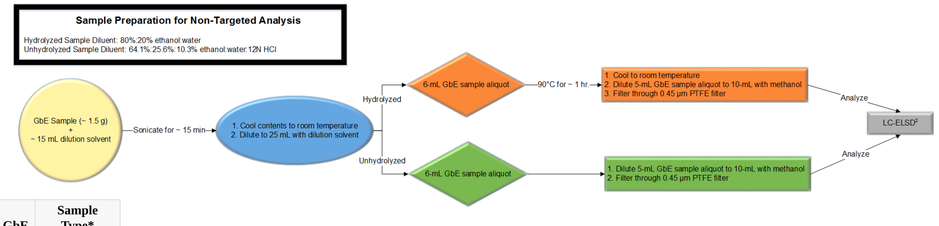
### Targeted LC/ Fluorescence Analysis[5]

- Samples were analyzed on a Shimadzu LC-2010C HT liquid chromatograph coupled to a Shimadzu RF-20AXS Spectrofluorometric detector with an excitation wavelength of 295 nm and an emission wavelength of 395 nm.
- Column: Phenomenex Intersil/InertClone 3 µ ODS-3, 100 Å, 150 x 4.6 mm ID.
- Mobile Phase: 5 mM aqueous potassium phosphate, with 5 mM aqueous sodium hexanesulfonate, pH adjusted to 2.5 with phosphoric acid (A) and acetonitrile (B) with a flow rate of 1.0 mL/min and the following gradient: 96%A, 4%B, hold 1 minute, then to 70%A, 30%B in 12 minutes, hold 7 minutes, then to 96%A, 4%B, in 1 minute, hold for 4 minutes. Total run time was 25 minutes.

*Evaporative Light Scattering detector

## Figure 1 – Representative GbE Samples

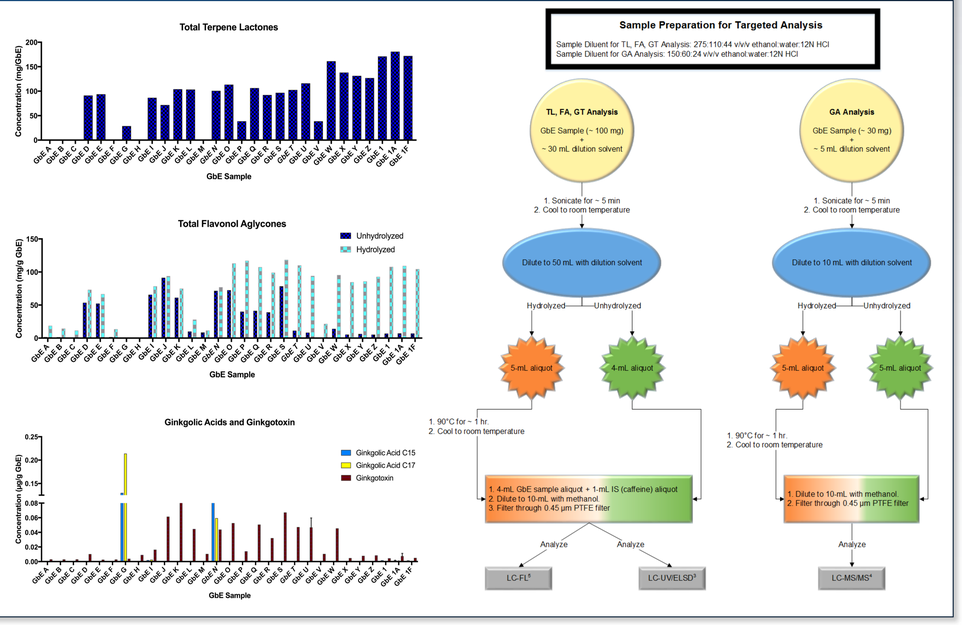

## Figure 2 – Non-Targeted Analysis




| GbE | Sample Type* |
|---|---|
| A | P.E. 24% |
| B | P.E. 10:1 |
| C | P.E. 4:1 |
| D | P.E. 24/6 |
| E | P.E. 24/6 |
| F | P.E. 24% |
| G | Leaf Powder |
| H | Leaf Extract |
| I | P.E. 24/6 |
| J | Extract™ |
| K | Dry Extract |
| L | EPG 246 |
| M | P.E. 4:1 |
| N | P.E. 24/6 |
| O | Extract 24/6 |
| P | Powder Extract |
| Q | Not Specified |
| R | Extract 24/6 |
| S | Extract USP |
| T | Extract 24/6/5 |
| U | NIST SRM |
| V | NIST Tablet SRM |
| W | Commercial Capsule |
| X | Commercial Tablet |
| Y | Commercial Tablet |
| Z | Commercial Tablet |
| 1 | NTP Tested Lot |
| 1A | NTP Tested Lot |
| 1F | NTP Tested Lot |

* P.E. = purified extract; ™ trademark extract; ratios are extraction ratios; percentages refer to flavonol glycoside, terpene lactone content.

## Figure 3 – Targeted Analysis




## Figure 4 – Authentication Representative Samples


## Table 1 – Authentication Results

| Samples not characteristic of G. biloba | |
|---|---|
| **GbE Sample** | Reported Sample Type |
| **GbE D** | Extract |
| **GbE E** | Extract |
| **GbE G** | Leaf Powder |
| **GbE I** | Extract |
| **GbE J** | Extract |
| **GbE K** | Extract |
| **GbE N** | Extract |
| **GbE O** | Extract |
| **GbE P** | Extract |
| **GbE Q** | Extract |
| **GbE R** | Extract |
| **GbE S** | Extract |
| **GbE W** | Extract (standardized capsule) |

| Samples are characteristic of G. biloba | |
|---|---|
| GbE Sample | Reported Sample Type |
| GbE L | Extract |
| GbE T | Extract |
| GbE U | Extract |
| GbE 1A | Extract |

**Authentication:**
Samples were run on silica gel 60, F254, HPTLC plates using two systems. System 1 consisted of ethyl acetate: acetic acid: formic acid: water: ADC2 [10/1.1/1.1/2.6] @35-40% humidity. Samples were run against reference standards of GbE (NIST SRM 3247), Genistein and a methanol solvent blank. TLC plates were visualized at 254 nm. System 2 consisted of toluene: ethyl acetate: formic acid: ADC2 [7/3/1] @35-40% humidity. Samples were run against reference standards of GbE (NISt SRM 3247), Genistein, Isorhamnetin, Quercetin, and a methanol blank. TLC plates were visualized with natural product reagent + PEG at 365 nm.

**Sample Preparation:**
A 0.3 g aliquot of each sample was diluted with 3 mL of 70% ethanol, sonicated and then heated at 70˚C for 30 minutes. Samples were analyzed using high-performance thin-layer chromatography (Table 4) by Alkemist Labs (Costa Mesa, CA).

## Summary and Conclusions

- Untargeted and targeted analyses of 31 GbE samples were conducted and selected samples were sent for HPTLC authentication analysis.
- Non-targeted analysis identified eleven GbE samples with large peaks in the flavonol aglycone region before hydrolysis, and could be used to flag GbE N as suspect while passing GbE T.
- Targeted analysis found that concentrations of marker compounds in each sample varied widely. Seven samples (A, B, C, F, G, H, and M) had concentrations above the limit of detection for only one or two of the twelve marker compounds. Targeted analysis found that the eleven samples flagged in the non-targeted assay, had high concentrations of quercetin and/or kaempferol prior to hydrolysis, suggesting that these samples may have been adulterated. In general targeted results showed that GbE samples making label claims about constituent content typically had more terpene lactones than the label claim, while flavonol content was typically in agreement with label claims.
- Authentication performed on 17 samples showed that only 4 had characteristics of an extract derived from *Ginkgo biloba* leaf. Authentication was not performed on 6 of the 7 samples with ≤ 2 markers present, the 4 tablet samples, or 2 of the 3 replicate NTP tested lot samples. If the 7 samples missing chromatographic markers of GbE are included with the 13 samples that failed authentication, 20 of 29 samples (70%) failed to meet requirements for authentic GbE samples.
- Non-targeted analysis flagged as suspect the same samples as the authentication analysis, with 11 of 13 samples found to be inauthentic also showing signs of potential adulteration.
- The NTP tested lot showed no signs of adulteration in the untargeted or targeted assays and was not flagged by the authentication assay. Its flavonol content was somewhat higher than the NIST SRM, but terpene lactone content was similar.

## References

1.Kressman, S., Müller, W.E., Blume, H.H. “Pharmaceutical quality of different *Ginkgo biloba* brands.”, J. Pharm. and Pharmacol. (2002), 54(5), 661-669.

2.van Nederkassel, A.M.; Vijverman, V.; Massart, D.L.; Vander Heyden, Y.; “Development of a *Ginkgo biloba* fingerprint chromatogram with UV and evaporative light scattering detection and optimization of the evaporative light scattering detector operating conditions.,” J. Chromatogr. A (2005), 1085, 230-239.

3.Gray, D.E.; Messer, D.; Porter, A.; Ferguson, S.; Harris, R.K.; Clark, A.P.; Algaier, J.W.; Overstreet, J.D.; Smith, C.S.; “Simultaneous quantification of terpenelactones and flavonol aglycones in hydrolyzed *Ginkgo biloba* extract by liquid chromatography with inline ultraviolet and evaporative light scattering detection.,: J. AOAC Int. (2005), 88(6), 1613-1620.

4.MRIGlobal Report, “Bulk Chemical Comprehensive Analysis of *Ginkgo biloba* L. powder extract, Chinese (Shanghai) Material, Amended Report II,” NTP ChemTask No. CHEM07256, MRIGlobal Assignment No. 615, submitted January 20, 2004.

5.Arenz, A.; Klein, M.; Fiehe, K.; Grob, J.; Drewke, C.; Hemscheidt, T.; Leistner, E.; “Occurrence of Neurotoxic 4′-*O*-Methylpyridoxine in *Ginkgo biloba* Leaves, Ginkgo Medications and Japanese Ginkgo Food.,: Planta Med. (1996), 62, 548-551.

6.NTP Technical Report: “Toxicology and Carcinogenesis Studies of *Ginkgo biloba* Extract in F344/N Rats and B6C3F1/N Mice.” NTP TR 578, NIH Publication No. 13-5920, March 2013.

## Acknowledgements

This work was funded through a contract with MRIGlobal from the Division of the National Toxicology Program within the U.S. Department of Health and Human Services, National Institutes of Health, National Institute of Environmental Health Sciences, Contract Award Number HHSN273201400020C.